Docket No.: 066654-0622 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Lipton, Stuart A., et al.	Customer No.: 41552
Appl. No.	:	09/876,187	Confirmation No.: 5845
Filed	:	June 05, 2001	
Title	:	METHODS OF DIFFERENTIATING AND PROTECTING CELLS BY MODULATING THE P38/MEF2 PATHWAY	
Grp./A.U.	:	1632	
Examiner:	:	Anne Marie Falk	

Declaration Pursuant to 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Stuart A. Lipton, declare as follows:

1) I am the Stuart A. Lipton who is named as a co-inventor on the above-identified patent application.

2) I understand that the claims stand rejected as allegedly lacking enablement.

3) Experimental results are presented herewith which corroborate the enablement of the claimed methods.

4) Mice were injected with a small number (50,000 cells) of control or MEF2CA-ES-derived neural progenitors labeled with bromodeoxyuridine (BrdU) along the anterior-posterior axis of the ipsilateral cortex one day after a 60-minute transient middle cerebral artery occlusion (tMCAO) (Exhibit 1, Figure 5-1). At 1 day post-grafting, the cells were uniformly and heavily labeled and clustered at the center of the injection site. Four weeks later, brain sections were stained with green fluorescent antibody (GFP) to identify the transplanted cells. Virtually all of the transplanted control enhanced green fluorescent protein (EGFP)-ES-derived neural

progenitors died in the ischemic tissue. In contrast, large numbers of the transplanted MEF2CA-ES-derived progenitor cells survived around the injection site and expressed the immature neuronal marker β-tubulin II (Exhibit 2, Figure 5-2). However, relatively few MEF2CA-ES-derived progenitor cells migrated out from the injection site in the ischemic core. These results show that MEF2CA-ES-derived neural progenitors can survive in ischemic mouse brain.

5) At 1 day post-transplantation, all of the grafted cells expressed EGFP. Then EGFP was down regulated in the majority of the cells in the days following engrafting. To better visualize the pattern and fate of engrafted cells *in vivo*, MEF2CA neural progenitor cells were labeled with cell tracker green (CTG) before transplantation. Cell Tracker Green can freely pass through cell membranes and, once inside a cell, undergoes a series of specific reactions, producing a cell-impermeant fluorescent dye that is susceptible to aldehyde fixatives. This probe is retained in living cells and is not transferred among adjacent cells in a population. Eight weeks later, mice brains were perfused and sectioned. Immunofluorescence labeling for green fluorescent antibody (GFP) to identify the transplanted cells was combined with labeling for NeuN in order to identify neurons (Exhibit 3, Figure 5-3). Note the NeuN immunoreactive neurons (red) forming the granule cell layer of the dentate gyrus. The yellow color indicates grafted cells adjacent to the lesion site doubled-labeled for NeuN (red) and GFP (green). No cells survived when they were transplanted close to the lesion cavity. Cells survived robustly when transplanted more medially into the tissue adjacent to the ischemic lesion. Representative images show differentiation of transplanted MEF2CA-ES-derived neural progenitor cells (green) into neurons (NeuN in red) at low (Exhibit 3, Figure 5-3), medium and high magnification (Exhibit 4, Figure 5-4). Low magnification shows the distribution of transplanted cells within the tissue. Medium magnification shows the migration of grafted cells in the ischemic area. The higher magnification insert confirmed the co-labeling of both markers in the same cell. The vast majority of cells migrating from the graft were neuronal, not astrocytic. These results show the distribution and differentiation of transplanted MEF2CA-NPC cells eight weeks post-implantation in adult mouse ischemic brain.

6) Following grafting of these genetically labeled, EGFP-expressing, MEF2CA-engineered neural precursors into the mouse ischemic brain, functional characterization of the

engrafted cells was performed by electrophysiology using patch electrodes and current clamp. EGFP-positive cells were detected with mostly neuronal morphologies in acute hippocampal slices eight weeks following transplantation. Action potentials were recorded, which were blocked by the addition of 1 μM tetrodotoxin (Exhibit 5, Figure 5-5A). Miniature excitatory postsynaptic currents, recorded under patch clump, indicated that the engrafted cells had made synaptic connections (Exhibit 5, Figure 5-5B). Fluorescence and IR-DIC imaging of live slices allowed for the targeted electrophysiological recordings from GFP-positive cells. No gross morphological malformations or damage to the hippocampus owing to the injection procedure was seen in any of the grafted animals. Data were collected from cells and recorded from brain slices that were prepared from a total of 10 animals. These results describe the electrical properties of transplanted MEF2CA-ES-derived cells.

7) In conclusion, these results show that transplanted MEF2CA neural stem cells survive, migrate and differentiate into neurons in the ischemic mouse cerebral cortex.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that any such willful false statement may jeopardize the validity of the application or any patent issued thereon.

2/22/2006
Date

Stuart A. Lipton, M.D., Ph.D.

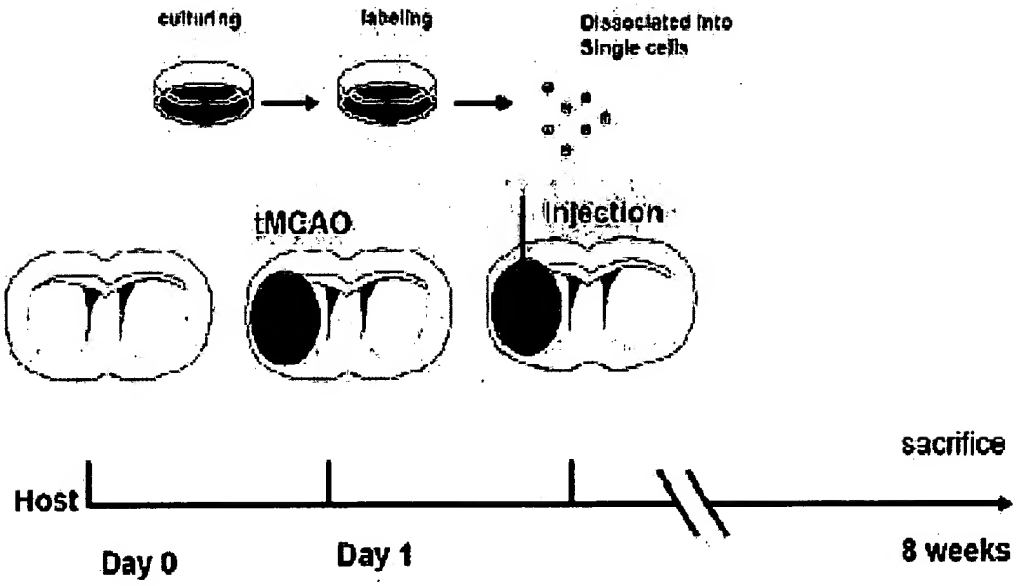

Figure 5-1: Time line for stem cell transplantation in a mouse model of stroke. The middle cerebral artery was occluded (MCAO) for 1 hour, followed by 24 hours reperfusion. The next day, MEF2CA-ES-derived cells or control EGFP-only-ES-derived cells were injected into the lesion site.

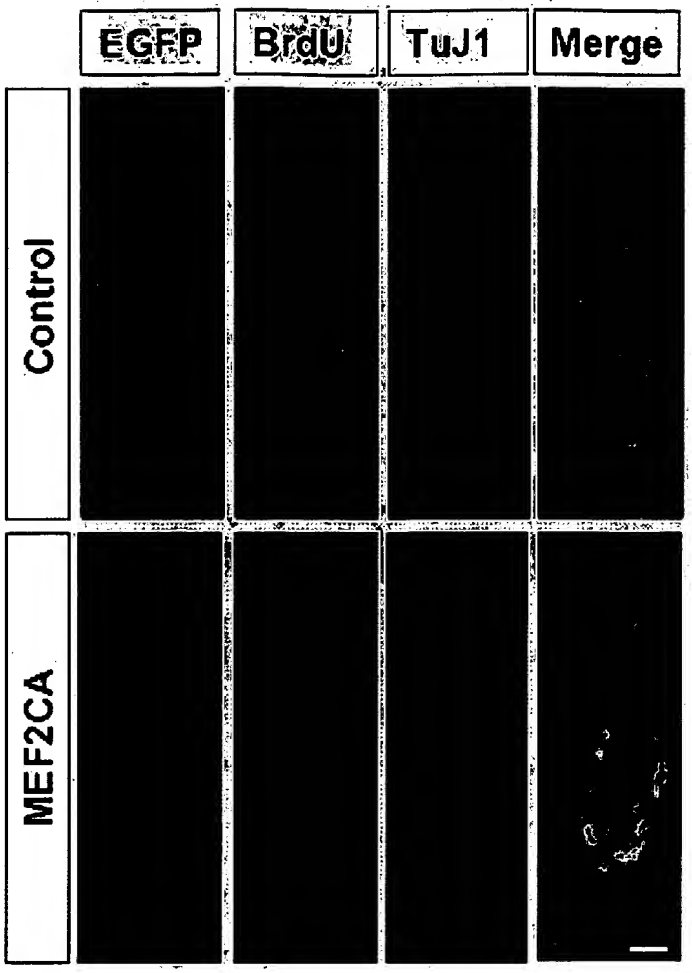

Figure 5-2. Neuronal differentiation of grafted MEF2CA-NSCs in acute mouse stroke model one month after transplantation.
Data showing distribution of transplanted cells one-month post-grafting. The vast majority of the transplanted MEF2CA-ES-derived progenitor cells expressed the early neuronal marker TuJ1. In contrast, virtually none of the EGFP-only cells expressed TuJ1. Scale bar is equivalent to 25 μm for all panels.

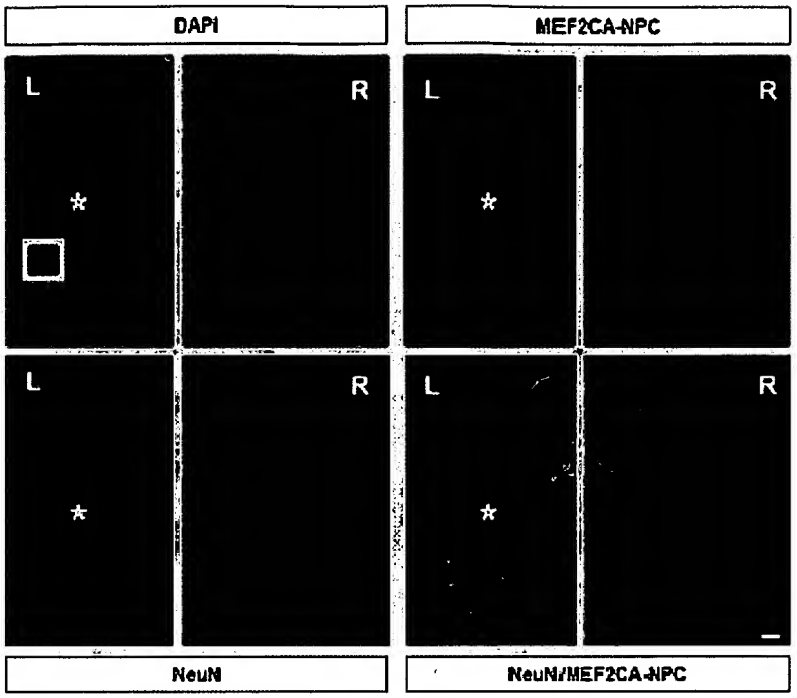

Figure 5-3. Distribution and differentiation of transplanted MEF2CANPC cells 8 weeks post-implantation in adult mouse ischemic brain. Representative images showing differentiation of transplanted MEF2CA-ES derived neural progenitor cells (green) into neurons (NeuN, red) at low power magnification (2x). Transplanted cells that differentiated into neurons were dual-labeled and thus yellow and are located outside of the ischemic core (*), where the cells had been injected. DAPI (blue) labeled cell nuclei. Scale bar is equivalent to 200 μm for all panels.

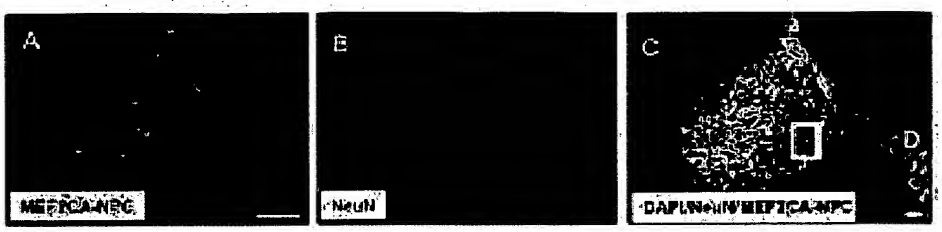

Figure 5-4. Distribution and differentiation of transplanted MEF2CANPC cells 8 weeks post-implantation in adult mouse ischemic brain at higher magnification.
(A-C) Medium magnification (10x) revealed widespread migration of engrafted MEF2CA-ES-derived neural progenitor cells into host brain parenchyma. *(D)* Higher magnification (box in *A*) confirmed co-labeling of transplanted neural progenitor cells (green) and NeuN (red), identifying the cells as engrafted neurons (yellow). Scale bar is equivalent to 200 μm for A, B, C (Inset, 15 μm).

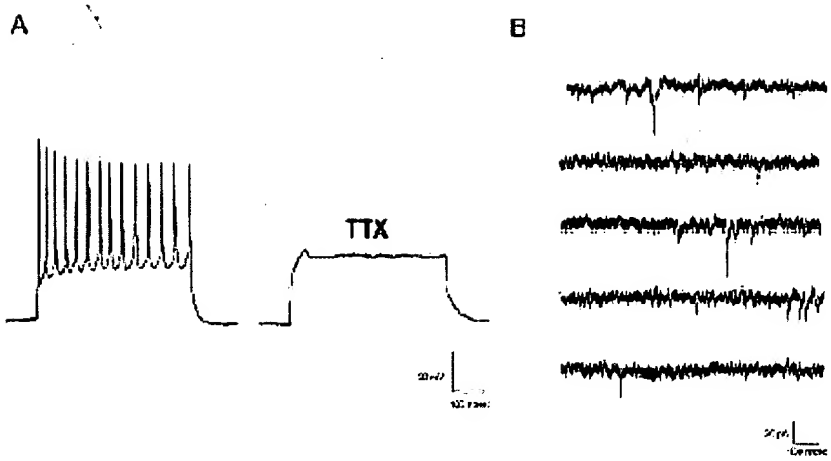

Figure 5-5. Functional characterization of a representative engrafted MEF2CA-NPC by electrophysiology. *(A)* Hippocampal slice recording under current clamp. Action potentials were present (left) and blocked by addition of tetrodotoxin (TTX). *(B)* The presence of miniature excitatory postsynaptic currents (mEPSCs) indicate that the engrafted cell been contacted to make a functional synapse.